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Monoclonal Antibodies for the Analysis of Gossypol in Cottonseed Products

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Immunogens, prepared by conjugating either (+)-gossypol or (−)-gossypol to *Limulus polyphemus* hemolymph protein, were used for immunization in the production of monoclonal antibodies. Hybridoma were evaluated for their relative affinity to racemic gossypol, (+)-gossypol, (−)-gossypol, gossypol analogues, and their lysine derivatives. The monoclonal antibody obtained showed higher affinity to gossypol and gossypol analogues as compared to their lysine derivative counterparts. An indirect competitive enzyme-linked immunosorbent assay (ELISA) with this antibody was used to measure gossypol in 15 cottonseed meal products; the results showed good correlation with results obtained using the AOCS (free gossypol) official method ($R^2 = 0.89$). The direct recognition of both free gossypol and bound gossypol using this antibody will be useful for rapid screening and quality control.

KEYWORDS: Gossypol; cotton; monoclonal antibodies; immunoassay; ELISA

INTRODUCTION

Gossypol is a polyphenolic aldehyde found in the roots, foliage, and seed of the cotton plant. During cottonseed and byproduct processing, gossypol will react with proteins to form bound gossypol. Bound gossypol has long been considered less toxic and less available than free gossypol when feeding animals. However, this is not always true depending on the treatment or the animals tested (*1*, *2*). Besides gossypol, other terpenoid aldehydes exist in cotton leaves, flower buds, and seeds (e.g., hemigossypol, hemigossypolone, heliocide H1, heliocide H2, heliocide H3, and heliocide H4). These terpenoid aldehydes exhibit some toxicity (*3*−*5*). The steric hindrance inhibits rotation about the internaphthyl bond giving rise to two chiral compounds: (+)-gossypol and (−)-gossypol. (−)-Gossypol has been found to have greater levels of certain biological activities, including enzyme inhibitory, antifertility, and toxic effects (*6*−*9*).

Because of gossypol's toxicity (*10*−*12*), the FDA has set a limit of 450 ppm of free gossypol in cottonseed products used for human consumption. The Protein Advisory Group of the Food and Agriculture Organization and the World Health Organization (FAO/WHO) set a limit of 600 ppm of free gossypol and 12 000 ppm total gossypol for human consumption. Commonly used methods for gossypol analyses include high-performance liquid chromatography (HPLC) (*13*−*15*) and the AOCS spectrophotometric methods (Ba 7-58, Ba 8-78). All of these analytical methods rely on defined extraction and derivatization procedures. The results from the HPLC and colorimetric methods do not always agree, because of their empirical nature and the existence of several types of bound gossypol. In addition, the AOCS method is laborious and is subject to interference from other terpenoid compounds that coexist in samples. The HPLC method requires strict sample preparation conditions.

We previously reported the development and use of anti-gossypol polyclonal antibodies for the analysis of gossypol (*16*), but sample derivatization was still required because the antibody only recognized gossypol in a conjugated form. This serum also showed relatively high affinity to gossypol degradation products, a characteristic that would yield false positives if this serum were employed for testing of complex biological samples. Gossypol is subject to oxidation, a factor that must be considered when synthesizing immunogens for antibody generation and in the development and interpretation of immunoanalytical methods.

The objective of this study was to produce monoclonal antibodies with high specific affinity to gossypol. An attempt was also made to produce antibodies specific for each of the two isomers, (+)- and (−)-gossypol. The Mab produced were characterized and compared with the AOCS method for the analysis of cottonseed meals.

MATERIALS AND METHODS

Supplies. Bovine serum albumin (BSA), *Limulus polyphemus* hemolymph (LPH), hydrogen peroxide, L-glutamine, sodium pyruvate, sterile cell culture penicillin, streptomycin, fetal bovine serum, 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid), Tween 20, NaCl, KH_2PO_4, Na_2HPO_4, ethanolamine, gossypol, sodium cyanoborohydride, goat anti-mouse peroxidase conjugated IgG + IgM (H + L), and Rosewell Park Memorial Institute 1640 (RPMI 1640) were purchased from Sigma Chemical Co. (St. Louis, MO). Citric acid (monohydrate granular) was from Mallinckrodt Inc. (Paris, KY). L-(+)-Lysine was from ACROS Organics (Pittsburgh, PA). Ribi Adjuvant System for mice was purchased from Ribi Immunochem Research, Inc. (Hamilton, MT). Poly(ethylene glycol) 1500 (PEG1500) was from Boehringer Mannheim

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GmbH (Germany). HAT (hypoxanthine−thymidine−aminopterin) and HT (hypoxanthine−thymidine) were bought as lyophilized, reconstitutable, 100× sterile solutions from Gibco BRL (Grand Island, NY). The myeloma cell line (NS-1, ATCC No. TIB 18) was from American Type Culture Collection (Rockville, MD). Tissue culture plates were from Costar (Cambridge, MA). Microtiter plates used for enzyme-linked immunosorbent assay (ELISA) were Immulon 2 HB (Dynex Technologies, Inc., Chantilly, VA). (±)-Gossypol was kindly donated by Dr. Peter Wan (USDA-ARS, New Orleans, LA); hemigossypol, 6,6′-dimethoxygossypol, and hexamethoxygossypol were kindly donated by Dr. Robert Stipanovic (USDA-ARS, College Station, TX). Phosphate-buffered saline (PBS, pH 7.3) solution contained NaCl (9 g/L), Na_2HPO_4 (1.11 g/L), and KH_2PO_4 (0.3 g/L) in distilled water. PBST was PBS with 0.5% Tween 20. Peroxidase substrate solution was made by adding 10 mg of 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) (ABTS) and 8 μL of 30% hydrogen peroxide solution in 24 mL of 0.1 M citrate buffer, pH 3.8. A mouse monoclonal isotyping kit (Roche Diagnostics Corp., Indianapolis, IN) was used to determine immunoglobulin isotype.

(±)-Gossypol, (+)-gossypol, (−)-gossypol, and gossypol analogue derivatives were made by mixing 1 part gossypol/analogue solution (2.5 mg/mL in methanol) and 9 parts of lysine solution (0.1 M in PBS), adding 0.3% $NaBH_3CN$ for 2 h at room temperature (Lane, 1975), and then storing at 4 °C less than 48 h prior to use. Underivatized (±)-gossypol, (+)-gossypol, (−)-gossypol, and gossypol analogue solutions (250 μg/mL) were made by mixing stock solution (2.5 mg/mL) with PBS instead of derivatizing solutions. To test the effect of aging on Mab recognition of standard solutions, (±)-gossypol, (−)-gossypol, and (+)-gossypol and their lysine derivatives were prepared (250 μg/mL in methanol) and stored at 4 °C in the dark for 6 weeks; these were then diluted for use in ELISA.

Cell Culture. Media were made using RPMI-1640 with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 unit/mL), 0.1 mg of streptomycin (0.1 mg/mL), and 0, 10, or 20% fetal bovine serum, for serum-free RPMI, RPMI-10, and RPMI-20 media, respectively. HT medium was RPMI-20 with sodium hypoxanthine (100 μM) and thymidine (16 μM). HAT medium was HT with the addition of aminopterin (0.4 μM). Conditioned medium, used during cell cloning, was supernatant from 3 days growth of myeloma cells in RPMI-20, filter-sterilized, and fortified with fresh fetal calf serum (20%), L-glutamine (2 mM), and sodium pyruvate (1 mM). During growth, all cell cultures were incubated at 37 °C with 5% CO_2 and 90−100% relative humidity. Live cells were counted after counterstaining with trypan blue (0.4% in PBS).

Synthesis of Protein Conjugates. Two LPH immunogens were made using either (+)-gossypol or (−)-gossypol that were purified by crystallization of racemic gossypol−acetic acid from acetone, according to the method of Dowd et al. (*17*). Purified (−)-gossypol or (+)-gossypol (5 mg) was dissolved in 2 mL of methanol, mixed with 15 mL of 1 mg/mL of LPH in PBS and stirred for 8 h under N_2 at room temperature in the dark. The reaction mixture was then dialyzed (MWCO 25K) 2 × 3 h in 1 L of 8 M urea, then 4 h with 2 L of 50 mM ammonium carbonate, and finally 4 h in 4 L of 25 mM ammonium carbonate. The product was lyophilized and stored at 4 °C in the dark.

Solid phase conjugate to be used in the ELISA was made using (±)-gossypol (3.9 mg) dissolved in 2 mL of ethanol. BSA (50 mg) was dissolved in 20 mL of PBS, and these two solutions were mixed with the addition of 60 mg of sodium cyanoborohydride ($NaBH_3CN$). The reaction was mixed at room temperature for 8 h under nitrogen in the dark, then dialyzed, and freeze-dried as described above.

Immunization. Six 1 month old, female, BALB/c mice were immunized with either (−)-gossypol or (+)-gossypol in Ribi adjuvant (0.5 mg/mL in PBS) as 0.1 mL s.c. and 0.1 mL i.p. per mouse. Booster injections were given every 3 weeks. One week after the fourth injection, blood (200 μL) was taken retro-orbitally to determine which mouse produced antibodies that bound to gossypol−BSA in an indirect noncompetitive ELISA. Selected mice were given one more injection prior to euthanasia and splenectomy. Animals were housed in the UW Animal Science Animal Care Facility under an approved institutional protocol. After four immunizations, six mice were tested for polyclonal antibody production and two were selected for fusion as they showed the greatest binding to coated (±)-gossypol−BSA in noncompetitive ELISA and showed inhibition at the lowest concentrations of (±)-gossypol in competitive ELISA.

Production of Hybridoma and Anti-Gossypol Mab. Three days after the last immunization, mice were euthanized by cervical dislocation and spleens were aseptically removed for fusion. Splenocytes from each spleen were fused with myeloma (5:1 ratio) using the fusion methods described in Plhak and Park (2003). Hybridoma from each fusion were suspended in 100 mL of HAT medium and cultured as 150 μL/well in 96 well tissue culture plates. After 1 week, fresh HT medium was added (100 μL/well). Cells were tested for antibody production on day 10 using gossypol−BSA solid phase in an indirect noncompetitive ELISA. Cells showing recognition were transferred to a 24 well plate and further subcultured in HT medium. Supernatants were screened using a competitive ELISA with (+)-gossypol, (−)-gossypol, and their lysine derivatives at concentrations of 25 and 0.0025 μg/mL (gossypol equivalent). One hundred thirty of 1340 wells from two fusions showed recognition to (±)-gossypol−BSA, and 14 wells among these 130 showed inhibition of antibody binding by either of the two gossypol isomers or gossypol−lysine. The cultures that showed recognition of free gossypol in competitive ELISA were cloned. After the fourth cloning, the Mab cell line (5C9D6E11E6) was obtained. Selected hybridoma were cloned by limiting dilution (0.1, 1, and 10 cells/well) in conditioned medium. Mab were produced by maintaining the Mab cell lines in RPMI-20, subculturing every 3 days, and pooling supernatants.

ELISA. A checkerboard ELISA was used to determine the amount of coating antigen gossypol−BSA and primary Mab needed in the ELISA (combinations chosen that gave absorbance ∼1). The competitive ELISA was as follows: 100 μL/well of 5 μg/mL of gossypol−BSA in PBS was coated on microtiter plate wells by incubation at 4 °C overnight. After the coating solution was removed by inverting the plate, blocking solution (200 μL/well of 0.5% BSA in PBS) was added. After 30 min at 37 °C, the solution was removed and washed with 3 × 200 μL PBST. Fifty microliters of serially diluted test standards (250, 25, 2.5, 0.25, 0.025, 0.00025, and 0 μg/mL of gossypol, gossypol analogues, or their lysine derivatives in 10% methanol were added followed by 50 μL of anti-gossypol solution (Mab cell culture supernatant diluted 1/10 in PBS). Following 30 min at 37 °C, the wells were washed using 3 × 200 μL PBST, and 100 μL/well of goat anti-mouse peroxidase conjugated IgG + IgM (diluted 1/6000 in 0.5% BSA in PBST) was added. After 30 min at 37 °C, the solution was removed and wells were washed with 3 × 200 μL PBST. ABTS substrate solution (100 μL/well) was added, and the absorbance at 405 nm was measured after 30 min in the dark at room temperature using a SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Cottonseed Meal Analysis. Fifteen cottonseed meal samples were obtained from Dr. Peter Wan (USDA SRRC, New Orleans, LA) who also provided corresponding data for free gossypol analyzed using AOCS method Ba 7-58 (gossypol levels from 0.079 to 1.088%), and total gossypol analyzed both by AOCS method Ba 8-78 and HPLC (*14*). For ELISA analysis, 2.5 mL of methanol was added to 0.1−0.13 g of finely ground cottonseed sample. This was vigorously shaken (30 min, dark) and centrifuged, and the supernatant was collected. The remaining residue was re-extracted with an additional 1.5 mL of methanol (30 min, dark), and the combined extracts were diluted (1/100 and 1/1000) with 10% methanol in PBS for ELISA analysis. Standards were tested in triplicate, and samples were determined using six replicates. Each plate included a gossypol standard curve. All experiments were repeated three times.

Statistical Analysis. I_{50} values (analyte concentration giving 50% reduction) were determined using SoftMAXPRO software, version 2.6 (Molecular Devices Corp.) based on the least-squares errors of the observed data in a four parameter equation. Standard analytes included gossypol and gossypol analogues and their derivatives. Analysis of variance and Fisher's protected least significant differences ($P = 0.05$, unless otherwise indicated) were calculated to compare I_{50} values using StatView software version 4.5 (Abacus Concepts Inc., Berkeley, CA). Liner regression was used to compare the results from ELISA and AOCS methods.

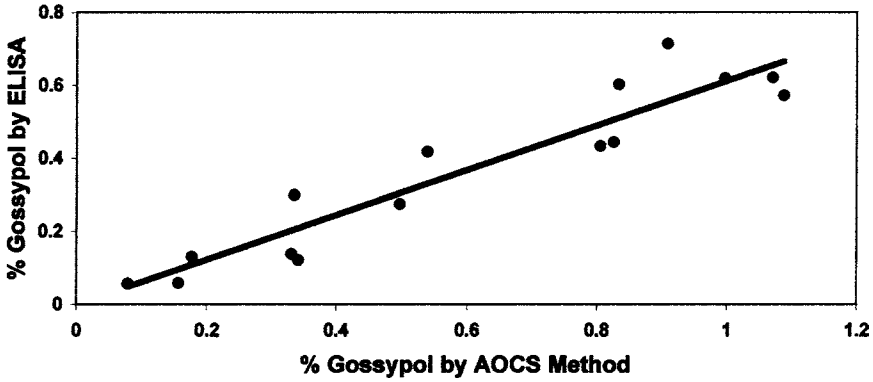

Figure 1. Correlation between ELISA and AOCS (free gossypol, Ba 7-58) methods using results obtained for gossypol analysis of cottonseed meal samples.

Table 1. Concentrations Required to Reduce a Blank Signal by 50% (I_{50} Values), Obtained from Competitive ELISA for Gossypol, Gossypol Isomers, and Analogues

compound	I_{50} (μM)
hemigossypol	0.185[a]
(±)-gossypol	0.494[ab]
(+)-gossypol	0.567[ab]
hemigossypol-lysine	0.651[ab]
(−)-gossypol	0.670[ab]
6,6-dimethoxy-gossypol	0.974[ab]
(+)-gossypol-lysine	0.988[ab]
(±)-gossypol-lysine	1.368[b]
(−)-gossypol-lysine	1.488[b]
6,6-dimethoxy-gossypol-lysine	6.840[c]

RESULTS AND DISCUSSION

To produce Mabs with specificity to gossypol, especially to the (−)-gossypol isomer and not degraded gossypol, we conjugated each gossypol isomer and modified the reaction conditions during conjugation to avoid gossypol degradation. Although immunizations were made with pure isomers of gossypol, of all the hybridoma obtained, no cultures showed preference to one isomer over the other.

The selected and cloned Mab was characterized for cross-reactivity toward (±)-gossypol, (+)-gossypol, (−)-gossypol, (±)-gossypol-lysine, (+)-gossypol-lysine, (−)-gossypol-lysine, as well as to hemigossypol, 6,6′-dimethoxygossypol, hemigossypol-lysine, or 6,6′-dimethoxygossypol-lysine (**Table 1**). In competitive ELISA, it recognized (+)-gossypol, (−)-gossypol, and racemic gossypol equally, indicating that it recognized only the naphthyl group and not the internaphthyl bond. The Mab showed a slightly higher affinity (lower I_{50} value) to hemigossypol as compared to gossypol, but the difference was not statistically significant ($P > 0.05$). Hemigossypol is equivalent to half of the structure of gossypol (one naphthyl group), and the smaller size may make it easier to diffuse to or fit the antibody binding site, increasing the relative affinity and resulting in a lower I_{50} value. The equal or slightly stronger affinity to hemigossypol is further evidence that the antibody does not recognize the internaphthyl bond.

Although one of our objectives was to obtain antibodies specific for each isomer and this was not achieved, equal cross-reactivity of the Mab to both isomers will still be useful in certain applications, e.g., to guide breeders in the development of gossypol-free cottonseed or for affinity purification of gossypol. The Mab was isotyped and found to be immunoglobin G_1, κ-light chain.

Lysine derivatization of gossypol, gossypol isomers, or some gossypol analogues decreased their binding affinity to the Mab. The Mab showed significantly lower relative affinity ($P < 0.05$) to each derivative when compared to the underivatized compound. For all compounds, except dimethoxygossypol, the I_{50} values approximately doubled as a result of derivatization. Poor solubility of dimethoxygossypol-lysine in methanol, although apparently clear (assessed visually), could have decreased the relative availability of the compound in solution, resulting in poor competition and a higher I_{50} value (approximately 10-fold higher).

Our previous study (*16*) developed polyclonal antibodies that showed higher relative affinity to gossypol degradation products formed in methanol after 6 weeks of storage at 5 °C. We hypothesized that similar products had been formed during synthesis of the immunogen and solid phase conjugates; therefore, in the current study, we used shorter reaction times, shorter dialysis times, and better protection from light and oxygen. These changes and the fact that we generated monoclonal antibodies prevented the generation/selection of antibodies with high relative affinity to products formed from gossypol in methanol during 6 weeks of storage at 5 °C (data not shown).

Figure 1 shows ELISA results for gossypol extracted from cottonseed meals by methanol vs those obtained for acetone extracts analyzed using AOCS method Ba 7-58 (measuring "free" gossypol, defined by the analytical parameters used). The correlation coefficient (R^2) was 0.89, indicating some similarity between these two analytical methods. The results obtained with the ELISA, however, were ~60% of those obtained for the same samples using the AOCS (free gossypol) method. The extraction solvents (methanol vs 70% acetone), solvent-to-seed ratios (~20:1 vs 50:1), number of repeated extractions (2 vs 1), and extraction times (1 h vs 2 × 30 min) used for the ELISA vs AOCS methods may have produced different extraction efficiencies. Methanol was selected for extraction in the ELISA method because of its ability to solvate gossypol and gossypol derivatives as well as its compatibility (at concentrations ≤ 10%) with ELISA reagents. Acetone, as used for the AOCS method, or acetonitrile (*18*) interfere with the activity of the protein reagents in the immunoassay and would require removal prior to analysis if used for sample extraction. The methanol extracts were analyzed immediately after extraction, but because of the relatively short half-life of gossypol in methanol (*15*), it is possible that some gossypol degradation occurred even during the time of the assay. The AOCS official method determines free gossypol by a colorimetric reaction of extracted aldehydic groups and aniline. The presence of nongossypol aldehydic compounds (e.g., lipid oxidation products) can result in overestimation of gossypol content (*15*, *19*) and possibly also

explains the higher results for gossypol concentration obtained using the AOCS method.

Determination of so-called "total gossypol", using either the AOCS (total gossypol) method or HPLC (*14*), measures that portion of gossypol that is extractable with 2-propanol and reactive with 3-amino-1-propanol. For the cottonseed meal samples used in this study, correlation between the ELISA results and the AOCS (total gossypol) and HPLC results was relatively poor, 0.39 and 0.44, respectively.

The Mab developed in this study can recognize gossypol directly, eliminating gossypol derivatization steps that are required for HPLC analysis, the AOCS method, and a previously reported ELISA (*16*). The Mab ELISA measures nonbound as well as some forms of extractable bound gossypol and shows high correlation with the AOCS (free gossypol) method. This new tool will be useful for rapid screening of gossypol-containing samples. The ability to recognize free and bound forms of gossypol simultaneously may make this method, in addition to alternative analysis methods, useful in understanding the potential biological role of free vs bound gossypol fractions.

CONCLUSIONS

Monoclonal antibodies that recognize both nonderivatized and derivitized gossypol were produced by immunizing mice with (+)- or (−)-gossypol−LPH conjugates. The Mab produced, used in ELISA, provides an alternative analytical method for gossypol analysis and can detect gossypol directly, without the requirement for gossypol derivatization, which is required for HPLC and AOCS methods. A rapid ELISA for monitoring gossypol in cottonseed products can now be envisioned. Application of this method for regulatory purposes, however, should consider the cross-reactivity when interpreting results.

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